Microtubule disassembly delays the G2–M transition in vertebrates

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When cell cultures in growth are treated with drugs that cause microtubules to disassemble, the mitotic index (MI) progressively increases as the cells accumulate in a C-mitosis. For many cell types, however, including rat kangaroo kidney PtK_1 cells, the MI does not increase during the first several hours of treatment [1–3] (Figure 1). This 'lag' implies either that cells are entering mitosis but rapidly escaping the block, or that they are delayed from entering division. To differentiate between these possibilities, we fixed PtK_1 cultures 0, 90 and 270 minutes after treatment with nocodazole, colcemid, lumi-colcemid, taxol or cytochalasin D. After 90 minutes, we found that the numbers of prophase cells in cultures treated with nocodazole or colcemid were reduced by ~80% relative to cultures treated with lumi-colcemid, cytochalasin D or taxol. Thus, destroying microtubules delays late G_2 cells from entering prophase and, as the MI does not increase during this time, existing prophase cells do not enter prometaphase. When mid-prophase cells were treated with nocodazole, the majority (70%) decondensed their chromosomes and returned to G_2 before re-entering and completing prophase 3–10 hours later. Thus, a pathway exists in vertebrates that delays the G_2–M transition when microtubules are disassembled during the terminal stages of G_2. As this pathway induces mid-prophase cells to transiently decondense their chromosomes, it is likely that it downregulates the cyclin A–cyclin-dependent kinase 2 (CDK2) complex, which is required in vertebrates for the early stages of prophase [4].

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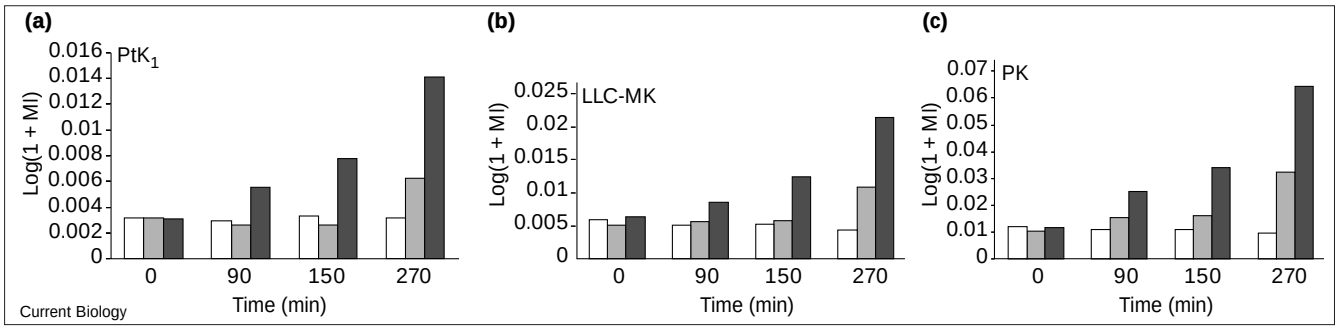
Results and discussion

To evaluate the possibility that the lag (see Figure 1) occurs because microtubule disassembly prolongs the prophase stage of mitosis [5], we determined the number of prophase cells in PtK_1 cultures following exposure to concentrations of nocodazole (10 μM) or colcemid (15 μM) that cause complete disassembly of interphase microtubules within 30 minutes, or to 1 μM nocodazole, which induces the disassembly of many but not all microtubules within 30 minutes (Figure 2). Our rationale was that, if disassembling microtubules prolongs prophase without retarding entry of G_2 cells into this stage of mitosis, then the number of prophase cells should be elevated in cultures fixed 90 minutes after exposure to nocodazole or colcemid. We chose PtK_1 cells for this study because they have a long and easily recognizable prophase and do not become committed to mitosis until well after chromosome condensation has been initiated [6].

Untreated PtK_1 cultures always contained more prophase than prometaphase/metaphase cells (Figure 3a,b) because, on average, the duration of prophase in PtK_1 (> 60 minutes [7]) is longer than the time from nuclear envelope breakdown to anaphase onset (52 minutes [8]). After 90 minutes in nocodazole (Figure 3a,b) or colcemid (Figure 3d) we found, however, that the number of prophase cells was severely reduced relative to controls, whereas after 270 minutes the number of prophases was similar to that of controls. Interestingly, when cultures were washed after a 90 minute exposure to 10 μM nocodazole, the number of prophase cells rapidly increased over the next 90 minutes to approximately twice that of untreated controls (Figure 3c). The number of prophase cells in cultures treated with the microtubule-stabilizing drug taxol, which arrests cells in mitosis without a lag (Figure 1), remained relatively constant over time (Figure 3e). Treating cultures with lumi-colcemid (Figure 3f), a non-functional analogue of colcemid [9], or cytochalasin D (Figure 3g), which inhibits actin filament dynamics [10], also failed to reduce the number of prophase cells.

From these data, we conclude that the failure of PtK_1 cells to accumulate in mitosis during the first several hours after microtubule disassembly arises from a transient depletion of prophase cells and from a failure of late G_2 cells to enter prophase. This transient delay in the G_2–M transition occurs specifically in response to disassembling microtubules, and it can be alleviated by allowing microtubules to reform. To our knowledge, this requirement of microtubules for a timely G_2–M transition has not previously been reported for higher organisms. The reversible nature of this G_2–M block provides a novel strategy for enhancing the number of prophase cells in growing cultures.

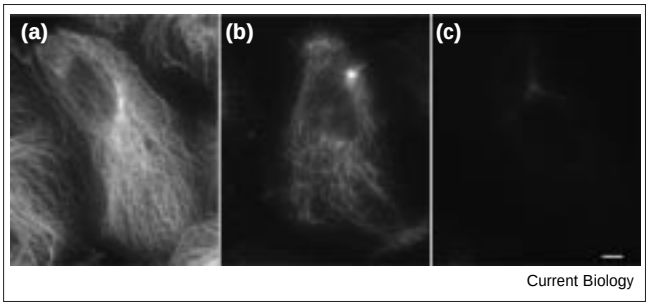
Figure 1



(a) PtK_1, (b) green monkey kidney (LLC-MK), and (c) pig kidney (PK) cultures were either untreated (white), or treated with nocodazole (light gray) or taxol (dark grey), and log(1 + MI) versus time (min) plotted. In contrast to taxol treatment, the MI in all of these cell types failed to increase during the first 150 min of nocodazole treatment.

Contrary to our findings, Jha *et al.* [3] did not see a lag when normal human cells were treated with nocodazole, although it was seen in response to colcemid or vinblastine (but not taxol) treatment. We reason that this was because the only nocodazole concentration tested (0.044 μg/ml or 0.15 μM) was below the threshold needed for disassembling microtubules [11]. Indeed, after a 30 minute treatment with 1 μM nocodazole, interphase PtK_1 cells still contained many microtubules (Figure 2b), and after 90 minutes these cultures contained more prophase and C-prometaphase cells than those treated with 10 μM nocodazole (compare Figure 3a with Figure 3b). Thus, in the absence of complete microtubule disassembly, late G_2 cells progressed into prophase with less of a delay than when microtubules were lacking. They then arrested in mitosis because even nanomolar concentrations of nocodazole inhibited the metaphase–anaphase transition by altering spindle microtubule dynamics [12]. As a result, for nocodazole, lowering the drug concentration actually diminishes the lag period — because the concentration required to arrest cells in mitosis does not deplete interphase cells of microtubules, which in turn allows them to progress unimpeded through the G_2–M transition.

Figure 2



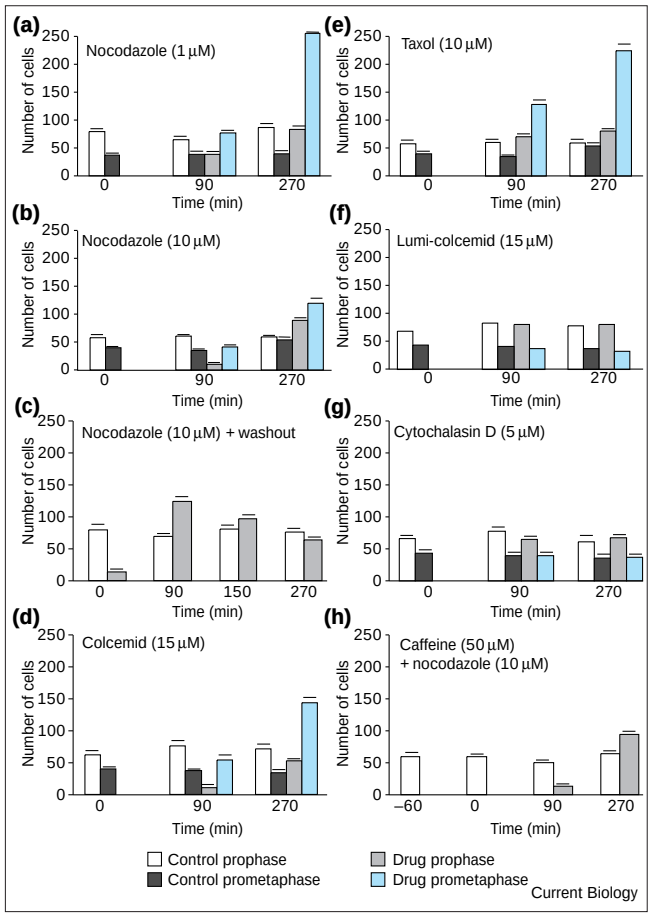
Numbers and distribution of microtubules during interphase, as revealed by indirect immunofluorescence of (a) untreated and (b,c) nocodazole-treated PtK_1 cells. (a) Untreated cells contained an extensive microtubule network, many of which persisted after a 30 min treatment with (b) 1 μM nocodazole. (c) After 30 min in 10 μM nocodazole, only a few short centrosome-associated microtubules were present. The scale bar in (c) represents 5 μm.

The term 'antephase' was coined in the mid 1900s to denote that period, just before the first signs of chromosome condensation, when cells are particularly sensitive to experimental insults [13]. Although it has been suggested that mitosis starts with antephase [14], a stronger argument can be made that mitosis starts only after the cell has become committed to the process [15]. In many cells, including PtK_1, this commitment does not occur until late prophase after chromosome condensation is well advanced [6]. Under this definition, the early to mid stages of chromosome condensation define the terminal phase of G_2, and provide a convenient, visible manifestation *in vivo* that the cell is undergoing the G_2–M transition.

What happens to the existing prophase cells after treating PtK_1 cultures with drugs that disassemble microtubules? Their numbers would be reduced if, in response to microtubule disassembly, cells in antephase were transiently inhibited from entering prophase to replace existing prophase cells as they entered C-prometaphase. Under this condition, however, the number of cells arrested in C-prometaphase would double during the first 90 minutes of drug exposure, which was clearly not the case (Figure 3b,d). Alternatively, the number of prophase cells would be reduced, and C-prometaphase cells would also fail to accumulate, if the sudden disassembly of microtubules induces early- to mid-prophase cells to return transiently to antephase, while also preventing antephase cells from entering prophase.

To evaluate this idea, we used video light microscopy (video-LM) to follow mid-prophase cells after exposing

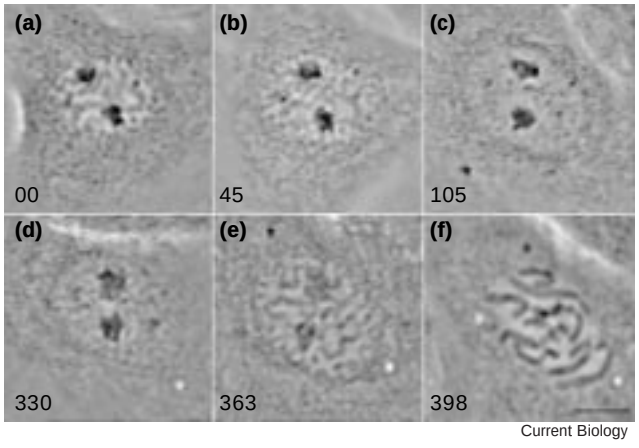
Figure 3



Number of prophase and/or prometaphase/metaphase cells per 5,000 cells, averaged over two separate experiments, after treatment with **(a)** 1 μM nocodazole, **(b)** 10 μM nocodazole, **(c)** 10 μM nocodazole followed by a wash at time = 0 min, **(d)** 15 μM colcemid, or **(e)** 10 μM taxol. In untreated cultures, the number of prophase cells was always slightly greater than the number of prometaphase/metaphase cells. After 90 min in nocodazole or colcemid, the number of prophase cells was significantly reduced even though there was (a) little or (b,d) no accumulation of C-prometaphase cells. In contrast, after (e) 90 min in taxol, prophase cells remained at control levels and there was a clear accumulation of cells arrested in C-prometaphase. (c) When cultures were treated with 10 μM nocodazole for 90 min and then washed, the number of prophase cells rapidly increased within 90 min to approximately twice that of untreated controls. The number of prophase cells in cultures treated with **(f)** lumi-colcemid or **(g)** cytochalasin D did not vary with time. **(h)** The number also did not vary with time after treating cultures with caffeine (50 μM), but it dropped dramatically when these cultures were then also treated with nocodazole. Each bar represents an average of two independent experiments. The line above each is the maximum of the two experiments, and the minimum would be an equal distance below the top of the bar.

them to 10 μM nocodazole. The illumination used during video LM could arrest progress through G_2 by triggering DNA-damage checkpoints, especially in cells lacking microtubules [7]. As for other cells, however, these checkpoints can be overridden in PtK_1 cells by pre-treating

Figure 4



Selected images from a time-lapse sequence of a PtK_1 cell, in a culture containing 50 μM caffeine, that was suddenly exposed during mid-prophase to nocodazole. In response to this treatment, **(a–c)** the cell transiently decondensed its chromosomes before **(d–f)** re-entering and completing prophase 5–6 h later. The elapsed time (min) is in the lower left corner of each image. The scale bar in (f) represents 10 μm.

cultures with 50 μM caffeine and, under these conditions, PtK_1 cells enter and complete prophase with normal kinetics when followed by video LM [7]. Thus, for our studies, we pre-treated PtK_1 cultures with caffeine 10–12 hours before exposing them to nocodazole. We found that, when mid-prophase cells were exposed to nocodazole, the majority (10/13) decondensed their chromosomes and returned to antephase before re-entering and completing prophase 3–10 hours later (Figure 4). The remainder (3/13) completed a prophase that was prolonged by about twofold (data not shown). Thus, the lag in the MI seen in response to disassembling microtubules is due to a transient drug-induced inhibition of the G_2–M transition: antephase cells are temporarily inhibited from progressing into visible prophase, and the majority of cells already in early- to mid-prophase at the time of treatment decondense their chromosomes and return to antephase before subsequently re-entering prophase several hours later.

A similar decondensation of chromosomes during mid-prophase occurs in PtK_1 cells when DNA-damage checkpoints are triggered by irradiation [6], but under these circumstances the cells fail to re-enter mitosis over a 3 day period [7]. We found that the transient reversion of prophase induced by microtubule disassembly was not inhibited by caffeine, which prevents the radiation-induced reversion of prophase cells. As caffeine targets the ATM kinase in the DNA-damage signaling cascade [16], the pathway delaying the G_2–M transition in response to microtubule disruption does not involve this kinase. It remains possible, however, that this pathway uses part of the existing DNA-damage checkpoint pathway, downstream from

ATM, to inhibit the activation of cyclin B–CDK1. Yet, as chromosome condensation in PtK_1 appears to be mediated by cyclin A–CDK2 [4], and because a commitment to mitosis does not occur in PtK_1 cells until nuclear cyclin B–CDK1 is explosively activated during late prophase [17], disrupting microtubules during mid-prophase is likely to return the cell to antephase by ultimately downregulating cyclin A–CDK2 activity.

Proper centrosome function may be required for cell-cycle progression [18] and the G_2–M transition [19], and disassembling microtubules clearly inhibits centrosome function. Evidence is also accumulating that the lag in the MI, seen when microtubules are disassembled, is a general feature of normal cells but is lacking in many transformed cells [3,20]. This raises the interesting possibility that disassembling microtubules during late G_2 triggers a cell-cycle checkpoint pathway that monitors microtubule or centrosome function. Clearly, this is an exciting area for future research.

Materials and methods

Cell culture

PtK_1 cell stocks were maintained in Ham's F-12 medium supplemented with 10% FBS; LLC-MK cell stocks were maintained in Eagle's MEM medium supplemented with 2% FBS and antibiotics; and PK cell stocks were maintained in DMEM supplemented with 10% FBS and antibiotics. Cells were trypsinized and plated onto 25×25 mm coverslips in 5 cm plastic Petri dishes. These coverslip cultures were then incubated in the dark at 37°C until they became mitotically active about 2 days later.

Mitotic index experiments

Taxol (10 μM), nocodazole (1 and 10 μM), cytochalasin D (5 μM), colcemid (15 μM) or β-lumi-colcemid (15 μM; generated by irradiating colcemid with 366 nm light until converted to β-lumi-colcemid [9]) were added directly to the media of growing coverslip cultures. Care was taken to maintain the cultures at 37°C at all times until the desired point of fixation. At 90, 150, and 270 min after adding the drug, two non-treated control and two drug-treated coverslip cultures were removed from the media and fixed in 1% glutaraldehyde in PHEM buffer. Cultures were then stained with Hoechst 33342. All coverslips were examined, and mitotic cells scored and counted, using a Nikon Optiphot light microscope equipped with a 60× 1.4 NA objective and a Quad flour epifluorescence attachment. The numbers of mitotic cells, and their stage in the mitotic cycle, was determined per 5,000 cells on each coverslip. A differential cell counter was used to keep track of the total number of cells, and also those that were in prophase, prometaphase/metaphase, anaphase, or C-prometaphase. Two coverslips (5,000 cells each) were used for each time point and the results averaged and plotted.

Video LM

The video LM conditions used in this study have been described [7]. In brief, Rose-chamber cultures of actively growing PtK_1 cells were treated with 50 μM caffeine for 10–12 h. They were then treated with conditioned medium containing 50 μM caffeine and 10 μM nocodazole. Several minutes later, a mid-prophase cell was located within the culture and its progress followed by phase contrast light microscopy. The video parameters used included shuttered 546 nm light obtained from a 50 W tungsten source, a framing rate of 4 min and an exposure of 1 sec per frame. All images were processed by NIH Image and stored on the hard drive.

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